ORIGINAL ARTICLE PARASITOLOGY

Rapid and sensitive diagnosis of *Acanthamoeba* keratitis by loop-mediated isothermal amplification

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Abstract

A loop-mediated isothermal amplification (LAMP) assay was developed for the detection of *Acanthamoeba*. The sensitivity of the LAMP assay was tested using different copies of positive DNA. The specificity of the assay was tested using DNA extracted from *Acanthamoeba*, *Pseudomonas aeruginosa*, *Candida albicans*, herpes simplex virus-1 and human corneal epithelial cells. Its effectiveness was evaluated and compared with culture, corneal smear examination and real-time PCR in corneal samples from mice with *Acanthamoeba* keratitis. We also tested three corneal samples from patients with suspected *Acanthamoeba* or fungal infection using LAMP. Loop-mediated isothermal amplification was confirmed to be very sensitive, with the lowest detection limit being ten copies/tube of *Acanthamoeba* DNA. The LAMP primers only amplified *Acanthamoeba* DNA. During the development of *Acanthamoeba* keratitis in mice, almost all of the positive rates of LAMP at each time post-infection were higher than those of culture or corneal smear examination. The total positive rate of LAMP was significantly higher than those of culture and corneal smear examination ($p < 0.05$), whereas the sensitivities of LAMP and real-time PCR were comparable. However, the trends of positive change in these different test methods were generally similar. Of the three clinical corneal specimens, two with suspected *Acanthamoeba* keratitis tested positive for *Acanthamoeba* using LAMP along with culture or corneal smear examination, whereas the other suspected fungal keratitis tested negative. The LAMP assay is a simple, rapid, highly specific and sensitive method for the diagnosis of keratitis caused by *Acanthamoeba*.

Keywords: *Acanthamoeba*, keratitis, loop-mediated isothermal amplification, sensitivity, specificity
Original Submission: 7 November 2012; **Revised Submission:** 21 December 2012; **Accepted:** 2 January 2013
Editor: M. Drancourt
Article published online: 12 January 2013
Clin Microbiol Infect 2013; **19:** 1042–1048
10.1111/1469-0691.12149

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Introduction

Acanthamoeba keratitis (AK), caused by the free-living amoeba *Acanthamoeba* spp., is a sight-threatening corneal infection that presents with corneal ulcerations [1]. In about 16% of cases, patients lose visual acuity even after treatment [2]. The incidence of AK appears to be increasing because of its frequent association with contact lens wear, which represents the cause of >85% of AK cases, especially in developed countries [3–5]. In developing countries, the main risk factors are corneal trauma and exposure to contaminated water [6,7]. With the increasing number of contact lens wearers all over the world, it is probable that the incidence of AK will continue to grow.

Early diagnosis of AK is important for effective treatment; however, the similarity of its clinical symptoms to herpetic and fungal infections makes this difficult to achieve [8,9]. Hence, AK is easily misdiagnosed in the clinic [10]. Laboratory examination for *Acanthamoeba* is an effective supplement for AK diagnosis, but the available *Acanthamoeba* tests, such as culture procedure and microscopic examinations of the corneal scraping and *in vivo* direct confocal microscopic examinations [11,12], are generally not sensitive, convenient or applicable enough for a precise diagnosis. PCR, especially real-time PCR, has been used to detect *Acanthamoeba* and has

shown higher sensitivity and a lower time cost than traditional techniques [13,14], but this technique is still not widely used because of the need for specialized instruments. Loop-mediated isothermal amplification (LAMP) is a recently developed molecular method that relies on autocycling strand-displacement DNA synthesis performed by the Bst DNA polymerase, large fragment. The main advantage of LAMP is its ability to amplify DNA sequences under isothermal conditions in the temperature range 63–65°C, thereby eliminating the need for a thermal cycler [15]. Moreover, four primers corresponding with six different sequences allow the assay to exhibit high specificity and efficiency.

In the present study, we constructed an LAMP assay to detect *Acanthamoeba* DNA and evaluated its specificity and sensitivity. The LAMP assay was also used for *Acanthamoeba* examination in the corneas of mice with AK, to study the association of the positive detection of *Acanthamoeba* DNA with AK development. Further, the validity of this method was confirmed in clinical specimens from patients with AK.

Materials and Methods

Acanthamoeba culture and preparation

The *Acanthamoeba* strains used in this study were isolated from ten eyes of patients with AK at the Shandong Eye Institute, and the genotypes of these isolates were all identified as T4 [7] in our previous study. The *Acanthamoeba* strains, stored at −80°C, were revived on 1.5% non-nutrient agar plates overlaid with 5 μL killed *Escherichia coli* at 37°C for 7 days, and then, the *Acanthamoeba* were inoculated with Peptone–Yeast–Glucose medium containing 500 U/mL penicillin and streptomycin for further culture at 27°C. After 5–7 days, 95% of the cultured *Acanthamoeba* were trophozoites. The amoebae were collected by centrifugation at 500 ***g*** for 7 min and washed three times with normal saline water.

Construction of AK mouse model

The AK model was constructed in ten groups of 6- to 8-week-old female BALB/c mice with ten different clinical *Acanthamoeba* strains. The Use of Animals in Ophthalmic and Vision Research by the Association for Research in Vision and Ophthalmology (ARVO) Statement was observed throughout the study, and the study was approved by the relevant institutional committees. Twenty mice in each group were anaesthetized, and their central corneal epithelia were scraped with a blade. A piece of filter paper with a diameter of 2.75 mm was placed onto the corneal surface, and 10 μL 3 × 10^6 copies/mL amoebae was inoculated into the eye. The eyelids were then sutured. After 24 hr of infection, the suture was taken out, and the filter paper was also removed. On days 1, 3, 5, 7, 10 and 15 post-infection, the ocular response to *Acanthamoeba* was examined with a slit lamp microscope. The scoring system used was essentially that described by Ren and Wu [16]. *Acanthamoeba* in the mouse corneas at different days after infection were tested by amoeba culture and direct smear examination of the corneal scraping, as well as by molecular methods.

DNA preparation

The cultured *Acanthamoeba* DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the protocol for cells. The total DNA from *Acanthamoeba*-infected mouse corneas and clinical corneal scrapings was also extracted using the kit. Positive DNA was prepared with the recombined plasmid pMD18T-Ac, which was constructed by inserting pMD18T (TaKaRa, Dalian, China) into a specific and conserved DNA sequence that had been amplified from isolated *Acanthamoeba* genomic DNA with primers F3 and B3. All of the extracted DNA quantification was performed by ultraviolet spectrophotometric measurement of the absorption at 260 nm on a sample of the DNA in solution.

LAMP reaction

The primers were designed using Primer Explorer V4 software (http://primerexplorer.jp/elamp4.0.0/index.html). A set of two outer and two inner primers that recognize six distinct regions on the target site (the conserved stem 29 region of the *Acanthamoeba* 18S rRNA gene) were used. The LAMP primer sequences are shown in Table 1. The two outer primers are described as forward outer primer (F3) and backward outer primer (B3), whereas the inner primers were described as forward inner primer (FIP) and backward inner primer (BIP). The LAMP reaction was performed using in-house prepared reaction mixtures, and each assay was conducted with 25 μL of the reaction mixture consisting of 1.0 μL each of the outer primers (5 μM), 1.0 μL each of the inner primers (40 μM), 2.5 μL of 10 × Bst DNA polymerase reaction buffer (New England Biolabs, Beverly, MA, USA), 3.0 μL of 50 mM $MgSO_4$, 5.0 μL of 5 M Betaine (Sigma-Aldrich, St Louis, MO, USA), 3.5 μL of

TABLE 1. Sequences of the primers used in the loop-mediated isothermal amplification and real-time PCR assays

Primer	Sequence
AC-FIP	5′-ATTACCCTAGTCCTCGCGCTTTTTG GGATAATGGAATAGGACCCT-3′
AC-BIP	5′-AATTGTCAGAGGTGAAATTCTTGGA TTTTTCCTTGGCAGATGCTTTC-3′
AC-F3	5′-CAATTTTCTGCCACCGAAT-3′
AC-B3	5′-CCCCTAACTTTCGTTCTTGA-3′
AC-F	5′-GGCCCAGATCGTTTACCGTGAA-3′
AC-R	5′-TCTCACAAGCTGCTAGGGGAGTCA-3′
AC-P	5′-GTCAGAGGTGAAATTCTTGG-3′

10 mM dNTPs, 1.0 μL of 8 U/μL Bst DNA polymerase (New England Biolabs), 2.0 μL target DNA and 4.0 μL ddH_2O. The reaction mixture was incubated in a water bath at 63°C for 1 h and heated at 80°C for 2 min to terminate the reaction. Positive and negative controls were included in each run. The amplified product was detected by adding 0.5 μL GeneFinder™ dye (Biovision, Xiamen, China) to each reaction tube. Yellowish-green fluorescence with naked eye observation indicated a positive reaction, and reddish orange indicated a negative reaction. In addition, the LAMP products were subjected to electrophoresis on a 1.5% agarose gel. After staining with ethidium bromide, the products were detected using UV light.

Sensitivity and specificity of the LAMP assay

The analytical sensitivity of the LAMP assay was tested using 0, 10^0, 10^1, 10^2, 10^3, 10^4, 10^5, 10^6 and 10^7 copies of positive DNA pMD18T-Ac. The copy number was calculated using the common formula with the mass and molecular weight of the plasmid. The specificity of the LAMP assay was evaluated using genomic DNA of *Acanthamoeba*, *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (ATCC 90029), herpes simplex virus-1 (HSV-1, McKrea strain) and human corneal epithelial cells (ATCC CRL-11135).

Real-time PCR

Real-time PCR was used to confirm the presence of *Acanthamoeba* DNA in the infected mouse corneas. Real-time PCR was performed using Taqman reagents and the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The specific primers and probes used in this assay (Table 1) were also designed based on the conserved stem 29 region of the *Acanthamoeba* 18S rRNA gene. The cycling conditions were 10 min at 95°C, followed by 45 two-step cycles (15 s at 95°C and 1 min at 60°C). The positive and negative controls were performed simultaneously.

Data analysis

Statistical analyses were performed using the paired-samples *t*-test; 95% CI were calculated using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA).

Results

Sensitivity and specificity of the LAMP assay for *Acanthamoeba*

The sensitivity of the LAMP assay for *Acanthamoeba* was determined by testing serial ten-fold dilutions of positive DNA pMD18T-Ac for which the copy numbers of amoeba DNA had previously been calculated. Positive reactions could be detected in tubes with ten or more copies of amoeba DNA by both electrophoresis assay and yellowish–green fluorescence observation (Fig. 1a). No amplification products were present in tubes with DNA concentrations below ten copies. The LAMP primers amplified only *Acanthamoeba* DNA. No LAMP products were detected with the DNAs of other common pathogens in the clinic, such as *P. aeruginosa*, *C. albicans*, HSV-1 or the host cell human corneal epithelial cells (Fig. 1b).

Clinical evaluation of AK models

All of the *Acanthamoeba*-infected mice developed clinical features of AK. The pictures of a typical infected mouse cornea at 0, 1, 3, 5, 7, 10 and 15 days post-infection are presented in Fig. 2(a). The clinical scores of all infected AK corneas were recorded with the aid of slit lamp examination and calculated at different days post-infection (Fig. 2b). Some infected corneas appeared to have slight corneal oedema at 1 day post-infection, and most corneas developed an obvious ulcer at 3 days. The AK was most severe at 5 days post-infection, with the features of eyelid tumidity, iris congestion, increasing new corneal vessels, enlarging inflammatory ulcers and whole cornea diffuse turbidity. After 5 days, the infected corneas were gradually alleviated with decreasing ulcers and repairs of scarring, and after 15 days, most of the inflammation was alleviated or healed.

Evaluation and comparison of LAMP and other methods for *Acanthamoeba* detection in AK mouse corneal samples

At 1, 3, 5, 7, 10 and 15 days post-infection, *Acanthamoeba* in AK mouse corneal samples were examined using LAMP assay, real-time PCR, and the other two most commonly used tests in clinic: amoeba culture and direct smear examination. For each test, one infectious eye was used for corneal scraping to culture; one, for smear examination; and one, for total DNA extraction. The detection results are shown in Table 2. The direct culture of the corneal scrapings in agar plates was the test with the lowest positive rate, only 50% were positive 3 days post-infection and all were negative at 15 days, for a total positive rate of 33.3%. Corneal scrapings smeared onto slides with 10% KOH for direct microscopic examination showed a higher positive rate than culture over each time-point, with 80% at 3 days post-infection, gradually decreasing to 20% at 15 days. The molecular methods used in this study present higher sensitivity than the above two methods. On the premise of an equal quantity of total infectious corneal DNA per reaction tube, the LAMP assay showed a higher positive detection rate (68.3%) than real-time PCR (61.7%), but the difference was not significant (p 0.24).

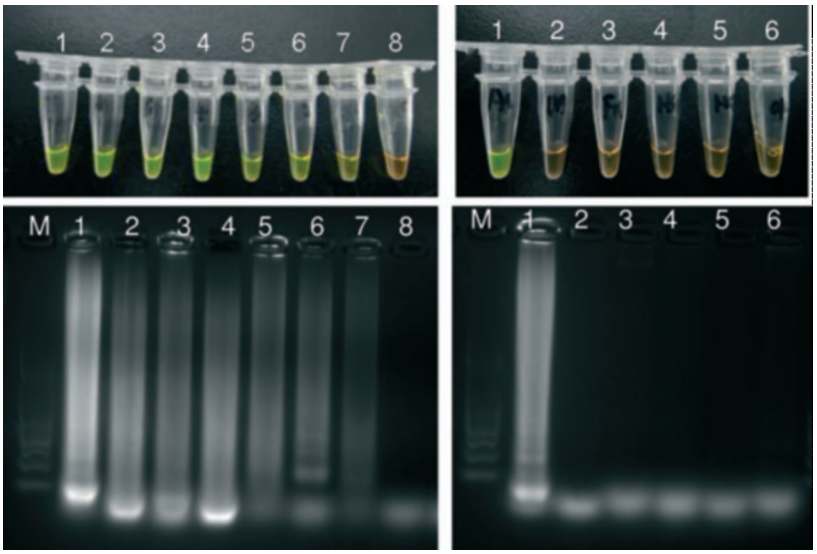

FIG. 1. Sensitivity (a) and specificity (b) of loop-mediated isothermal amplification (LAMP) for the detection of *Acanthamoeba*. The LAMP products were assessed by visual detection after being added to GeneFinder dye (top panels) and agarose gel electrophoresis (lower panels). In (a). 1: 10^6 copies/tube; 2: 10^5 copies/tube; 3: 10^4 copies/tube; 4: 10^3 copies/tube; 5: 10^2 copies/tube; 6: 10^1 copies/tube; 7: 10^0 copies/tube; 8: negative control without target DNA. In (b) 1: *Acanthamoeba*; 2: *Pseudomonas aeruginosa*; 3: *Candida albicans*, 4: herpes simplex virus-1, 5: human corneal epithelial cell; 6: negative control without target DNA. M: DNA Marker.

However, the positive rate of the LAMP assay was significantly different than that of culture (p 0.0004) and smear examination (p 0.01). The positive rate of LAMP assay for amoeba detection was 90% at 3 and 5 days post-infection and even 30% at 15 days.

Confirmation of the LAMP assay in clinical AK corneal specimens

The LAMP assay was also applied to detect *Acanthamoeba* in clinical corneal specimens to evaluate its diagnostic effectiveness. The following three clinical corneal specimens were subject to DNA extraction: the first, a piece of the corneal button taken from a patient with definite AK during keratoplasty; the second, some corneal scrapings also from a patient with definite AK; and the third, some corneal scrapings from a patient with definite fungal keratitis. The *Acanthamoeba* DNA detection results using LAMP are shown in Fig. 3. The corneal specimens from the two patients with AK generated positive reactions, along with the positive control, whereas the specimen from patient with fungal keratitis demonstrated no amplification, nor did the negative control.

Discussion

Clinical suspicion is the first and most vital step in diagnosis of AK, but the early clinical appearance of the disease usually mimics herpetic and fungal infections [8,9], easily leading to misdiagnosis and delaying effective treatment. Cultures used in conjunction with corneal smears for microscopy are the most common method for the laboratory diagnosis of AK; however, the culture is time-consuming, and the corneal smear examination for *Acanthamoeba* depends on experienced technicians. From the microscopic pictures of the clinical AK corneal smears in our laboratory (see Supplementary material, Figure S1), it was found that trophozoites or cysts of *Acanthamoeba* were often similar to the scraped cells or cell debris. Confocal microscopy has recently become a powerful tool for rapid diagnosis of the infection *in vivo*, and without the need to wait for culture and microbiological analysis [11,12], but this method requires a specialized, expensive apparatus, which is not suitable for all laboratories. Therefore, development of a sensitive, convenient and rapid method for laboratory detection of *Acanthamoeba* to assist AK diagnosis is urgently required.

PCR has been widely used in the detection of various pathogens, including *Acanthamoeba* [13,14,17,18], because of its relatively high sensitivity and effectiveness, but PCR requires precise equipment, such as a thermal cycler for the amplification of DNA, which limits its application in the clinic. However, the LAMP assay is a simple diagnostic tool that can be completed in a regular laboratory water bath within 70 min, and the final amplified product of the LAMP assay can be detected through unaided visual examination by adding GeneFinder to the reaction tube. Moreover, we have shown that

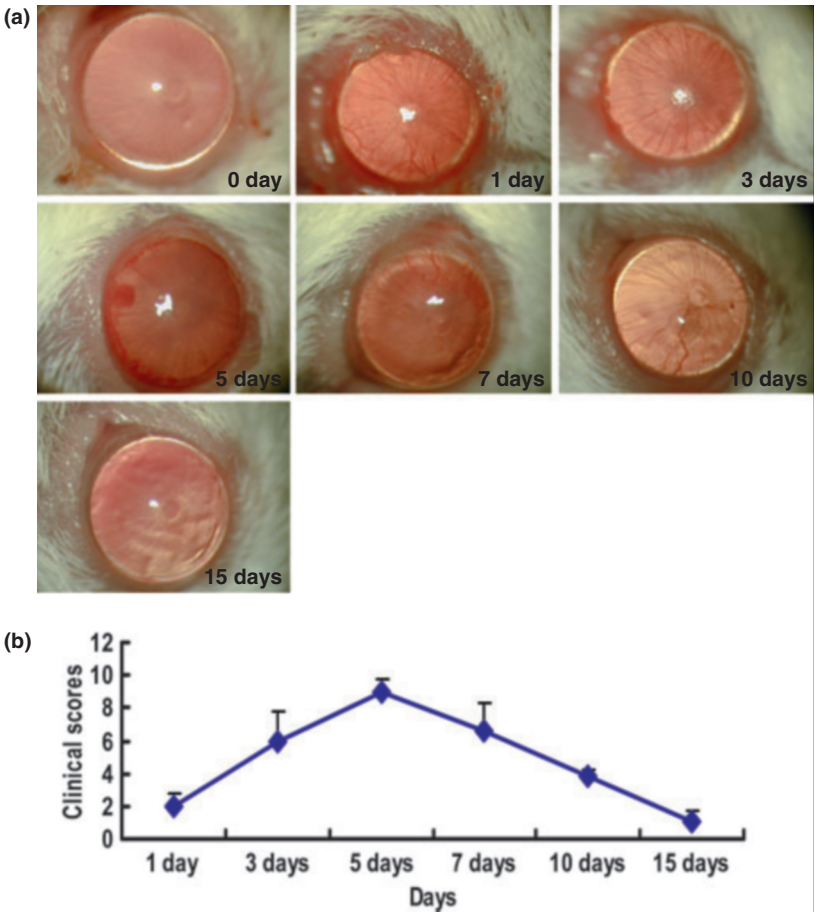

FIG. 2. Construction of *Acanthamoeba* keratitis in BALB/c mice. The mouse corneal epithelia were scraped and inoculated with 3×10^6 *Acanthamoeba* and covered with a filter paper on the cornea surface. At 1, 3, 5, 7, 10 and 15 days post-infection, the ocular response to *Acanthamoeba* challenge was examined with a slit lamp microscope (a), and the clinical scores were recorded (b). Corneas developed obvious ulcers at 3 days. At 5 days, the ulcers developed to be more severe, and after 5 days, the infectious corneas were gradually alleviated.

TABLE 2. Comparison of loop-mediated isothermal amplification (LAMP) with other detection methods for *Acanthamoeba* detection in corneal samples from mice with *Acanthamoeba* keratitis

	Culture			Smear			LAMP			Real-time PCR		
Day	+	–	Positive rate (%)	+	–	Positive rate (%)	+	–	Positive rate (%)	+	–	Positive rate (%)
1	4	6	40	7	3	70	8	2	80	8	2	80
3	6	4	60	8	2	80	9	1	90	9	1	90
5	4	6	40	7	3	70	9	1	90	7	3	70
7	3	7	30	5	5	50	7	3	70	5	5	50
10	3	7	30	5	5	50	5	5	50	6	4	60
15	0	10	0	2	8	20	3	7	40	2	8	20
Total	20	40	33.3	34	26	56.7	41	19	68.3	37	23	61.7

+, positive; –, negative.

LAMP is also highly specific and sensitive, as it could detect as few as ten copies of HSV-1 DNA [19,20]. The LAMP assay has been used for the detection of microbes in many regions, but at present, there has been no report on LAMP for *Acanthamoeba* detection. In this study, we developed a LAMP assay for the detection of *Acanthamoeba* and evaluated its specificity and sensitivity. This assay specifically amplified only *Acanthamoeba* DNA, and no cross-reactivity was observed with other

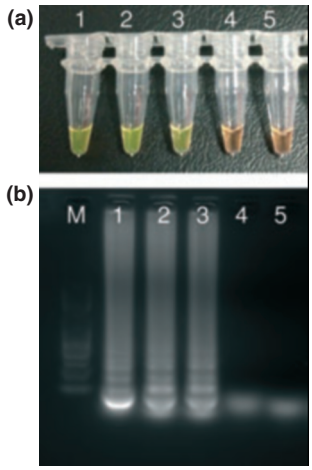

FIG. 3. Detection of *Acanthamoeba* in corneal specimens using the loop-mediated isothermal amplification (LAMP) assay. Three clinical corneal specimens were subject to the LAMP assay for *Acanthamoeba*, and the amplification was assessed by visual detection after being added to GeneFinder dye (a) and agarose gel electrophoresis (b). 1: *Acanthamoeba*-positive DNA; 2: DNA extracted from a corneal button of a patient with suspected *Acanthamoeba* keratitis during keratoplasty; 3: DNA from corneal scrapings of a patient with suspected *Acanthamoeba* keratitis; 4: DNA form corneal scrapings of a patient with suspected fungal keratitis; 5: negative control without target DNA. M: DNA Marker.

common pathogens that may cause eye infections, such as HSV-1, *P. aeruginosa* or *C. albicans*. It also had almost the same sensitivity as real-time PCR, as the lower detection limit of the assay was ten copies of DNA/tube.

In the present study, we compared the dynamic positive rate of the LAMP assay for *Acanthamoeba* detection in AK mouse corneal samples with that of real-time PCR and two other common test methods. We found that the total positive rate of LAMP was significantly higher than that of culture or corneal smear examination; however, the sensitivity of LAMP and real-time PCR was comparable. Almost all of the positive rates of LAMP at each time point post-infection were higher than those of culture or corneal smear examinations. However, the trends of positive change in these different test methods were generally similar. In addition, it is worth mentioning that we constructed AK mouse models as previously reported [16], but with the difference in treatment being that we used filter paper instead of the contact lens to overlap the corneal surface after epithelial scraping. The success rate of inducing AK in the BALB/c mice was almost 100%. The induced AK in this study displayed obvious inflammation at 3 days post-infection and was most serious at 5 days post-infection; after that, the inflammation decreased gradually and had almost returned to the normal level after 15 days. The corresponding positive rate of the LAMP assay was highest at 3 days post-infection and gradually decreased after that. *Acanthamoeba* DNA could be detected even at 15 days post-infection, indicating the residual DNA that the host had not eliminated. It is clear that most of the load of *Acanthamoeba* in the infected cornea had appeared before the inflammation became most serious. As the inflammatory reaction was also the process of the host eliminating the invading pathogen, the load of *Acanthamoeba* was reduced after the most severe portion of the reaction.

In this study, the LAMP assay was also applied to detect *Acanthamoeba* in clinical corneal specimens, and the detection results were consistent with the clinical diagnosis. The LAMP assay was positive for the detection of *Acanthamoeba* in a corneal button from a patient infected with *Acanthamoeba* for about 40 days whose cornea was seriously damaged and required keratoplasty. The corneal smear examination was also positive, while the culture appeared negative, probably because of previous drug usage. Regarding the corneal scrapings from a patient infected with *Acanthamoeba* for about 16 days whose cornea developed visible ulcers, the LAMP assay was positive for *Acanthamoeba* detection, along with culture and smear examination. The developmental course of the human cornea infected with *Acanthamoeba* was slower than that of the mouse cornea, and the ability to self-repair was much weaker. Our study found that only if there was an inflammatory reaction after *Acanthamoeba* infection in cornea could *Acanthamoeba* DNA be detected, despite the length of infection time. Of course, this conclusion should be further confirmed with a larger sample size.

In summary, we developed an LAMP assay for *Acanthamoeba* detection in infected corneal specimens, and we found that the sensitivity of the LAMP assay was significantly higher than that of commonly used test methods, such as culture and corneal smear examination, and comparable even with real-time PCR. Beyond its relatively high sensitivity and specificity, the LAMP reaction is simple and highly effective. Therefore, LAMP is worth considering for clinical application as a laboratory test method for *Acanthamoeba* examination to aid in AK diagnosis.

Acknowledgement

This study was supported by the National Natural Science Foundation of China (81100651) and by the Fund of the Academy of Medical Science of Shandong (2010-22).

Transparency Declaration

The authors declare no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Microscopic examination of the clinical *Acanthamoeba* keratitis corneal smear. Corneal scrapings were smeared onto a slide with 10% KOH for direct microscopic examination (× 400). The arrows point to two different morphologies of *Acanthamoeba*, trophozoite and cyst.

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